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| 14. ABSTRACT A major challenge in prostate cancer research is to develop novel therapies that can delay or prevent the progression of androgen-sensitive prostate cancer to androgen-independence. Androgen receptor (AR) is often overexpressed and plays an essential role in androgen-refractory prostate tumors. Our preliminary studies suggest that heat shock protein 90 (Hsp90) is required for androgen-independent AR nuclear localization in androgen-refractory prostate cancer cells. This project tested our research hypothesis that Hsp90 plays a critical role in ligand-independent AR nuclear localization, an essential step leading to androgen-refractory prostate cancer. During the funding period, we showed that Hsp90 deacetylation by HDAC6 is a critical step involved in castration resistance of prostate cancer cells. | | | | | |
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Introduction

Prostate cancer (CaP) is the most common cancer and the second most common cause of cancer death among men in the United States (1). The androgen deprivation therapy (ADT) is the standard treatment for metastatic CaP. However, patients invariably recur with hormone-refractory or androgen-independent (A-I) CaPs. Effective treatment for A-I CaP is desperately needed for this lethal disease. Recent studies showed that, although the A-I tumors are androgen depletion independent, their growth is androgen receptor (AR)-dependent (2,3). Novel strategies to inhibit ligand-independent AR activity may be effective against A-I prostate tumors. Androgen receptor (AR), a member of the steroid receptor superfamily, is a ligand-dependent transcription factor that controls the expression of androgen-responsive genes (4). Intracellular trafficking is an important mechanism in the regulation of many transcription factors, including AR. In order to access its target genes, a transcription factor requires localization to the nucleus. Likewise, retention of a transcription factor in the cytoplasm prevents its activity. Thus, a key regulatory step in the action of AR is its nuclear translocation. In androgen-sensitive cells, AR is localized to the cytoplasm in the absence of ligand. Upon addition of androgens, AR translocates to the nucleus and transactivates target genes. However, in A-I prostate cancer cells, AR remains in the nucleus even in the absence of androgen and transactivates androgen-responsive genes, leading to A-I growth of prostate tumors (2,3). Our preliminary studies demonstrate that the Hsp90 inhibitor, 17-AAG, prevents A-I nuclear localization and activation of AR in the androgen-refractory C4-2 cells (5). This study will further determine the role of Hsp90 in ligand-independent AR nuclear localization and prostate cancer progression to A-I using the C4-2 androgen-refractory prostate cancer cell line as a model.

Body

Task 1: Determining the effect of HSP90 level on AR nuclear localization in the absence of androgen (months 1-18).

- A. Develop lenti-viral expression vector for HSP90 protein and siRNA specific for HSP90 (months 1-10)
- B. Infect LNCaP cells with HSP90 expression lenti-virus and assay for the effect of HSP90 overexpression on AR intracellular localization (months 10-18)
- C. Infect C4-2 cells with HSP90 siRNA lenti-virus to assay the effect of HSP90 downregulation on the intracellular localization of AR (months 10-18)

Lentiviral expression vectors are chosen to express Hsp90 protein and siRNA in this project because they offer high infection efficiency. We have purchased lentiviral vectors pCDH cDNA cloning vector (Cat# CD511A-1) and pSIH-H1 shRNA cloning vector (Cat# SI500A-1) from System Biosciences (SBI) (**Fig. 1**). Both vectors express green fluorescence protein (GFP), which allows us to monitor the infection efficiency conveniently. We have generated one lenti-viral vector that expresses Hsp90 protein and another one that expresses siRNA specific for Hsp90. Unfortunately, the lenti-viral Hsp90 protein expression vector had a stop codon in the Hsp90 open reading frame, as indicated in our previous progress report. We have recently corrected the mutation in the expression vector. We are in the process of generating and characterizing pseudoviral particles for Hsp90 overexpression.

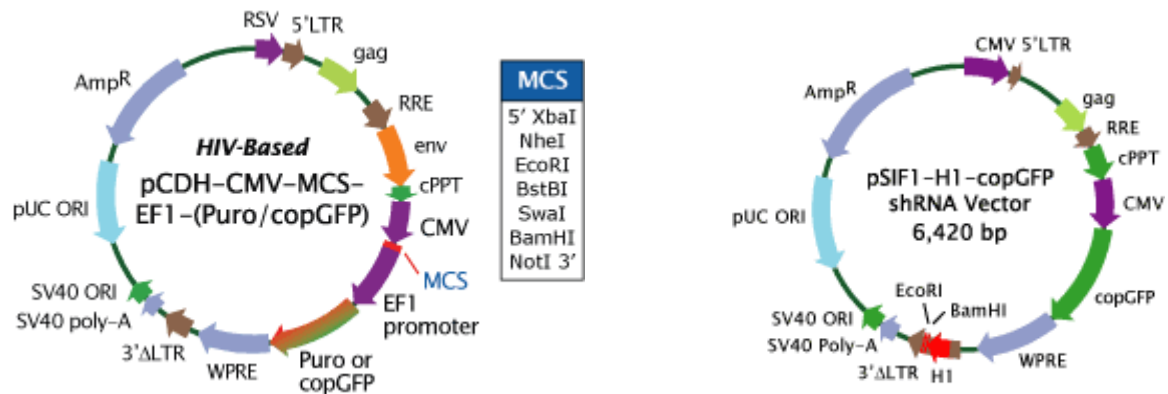


Fig. 1. Lenti-viral vectors. Left diagram shows the pCDH cDNA cloning vector (Cat# CD511A-1) for Hsp90 overexpression. This vector includes a CMV promoter before the multiple cloning sites and an EF1 promoter just before a green fluorescence protein (GFP) as a reporter for transduced cells. Right diagram shows the knockdown lentivector, pSIF1-H1 shRNA cloning vector (Cat# SI500A-1). This vector includes a CMV promoter for constitutive expression of GFP and an RNA polymerase III promoter to drive the expression of the siRNA sequence. Both vectors were purchased from System Biosciences (SBI) Mountain View California.

We have designed siRNA sequence using Integrated DNA Technologies RNAi online software tool (IDT, Coralville, IA). The sequence of siRNA specific for Hsp90 is 5'GGACCAGGTAGCTAACTCA3'.

To generate Hsp90 expression vector, the human Hsp90 cDNA sequence was PCR amplified using forward (For) and reverse (Rev) primers with indicated sequences:

For: 5' CAGGCTAGCACCATGCCTGAGGAAACCCAGACCCA 3'

Rev: 5' TCAGGCGGCCGCTTAGTCTACTTCTTCCATGCG 3'.

The amplified cDNA was restriction digested and cloned into the multiple cloning sites (MCS).

Lentiviral vectors were next packaged into packaging cell line 293TN (SBI, California). An RSV-5LTR or CMV-5LTR is included in each of the vectors for expression of constructs into the producer cell line (293TN). The pPACK H1 plasmids: pPACKH1-GAG, pPACKH1-REV, and PVSV-G provide all the necessary elements to produce VSV-G pseudoviral particles. These particles provide the most efficient method for high titer transduction into target cells.

The siHsp90 construct was sequence verified and packaged. The pseudoviral particles were transduced into C4-2 prostate cancer cells. Based on GFP expression, about 60% of the cultured C4-2 cells were transduced by siHsp90 lenti-viral particles in our experiments. We have isolated siHsp90 vector transduced C4-2 cells by fluorescence activated cell sorting (FACS) analysis. **Fig. 2** shows that FACS sorted C4-2 cells are positive with GFP expression. These GFP-positive cells were used to determine if siHsp90 lenti-viral infection is effective in down-regulating Hsp90 expression. The data thus far are very promising.

The down-regulation of Hsp90 mRNA by siRNA specific for Hsp90 was revealed by real-time PCR (**Fig. 3**). The primer pair used for Real-time PCR are 5' TCTGGAAGATCCCCAGACAC 3' (Forward) and 5' AGTCATCCCTCAGCCAGAGA 3' (Reverse).

Down-regulation of Hsp90 protein by siRNA in C4-2 cells was reproducibly observed in Western blot analyses using anti-Hsp90 antibody (**Fig. 4**). This result indicates that our siRNA lenti-viral vector is effective in down-regulating Hsp90 protein expression. Also, the expression of PSA was down-regulated in C4-2 cells with shHSP90 expression, supporting our research hypothesis that HSP90 enhances AR activity. However, the HSP90 levels rebounded after a few more passages (data not shown). We are trying to overcome this technical problem by investigating the timing and possible reasons of HSP90 rebound in C4-2 expressing shHSP90.

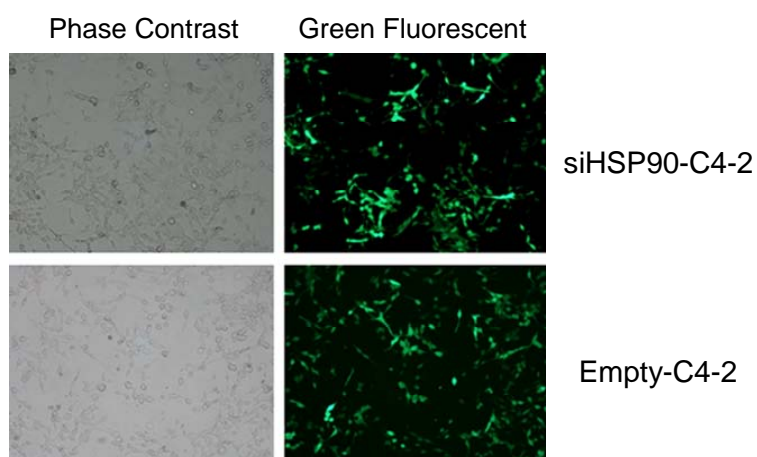


Fig. 2. C4-2 Cells Expressing GFP and siHsp90 Lentiviral Vector. Lentiviral Expression vector was packaged into 293TN cells. The viral particles were then used to infect parental C42 cells. Lentiviral vector was transduced and integrated into the genomic material of the parental cells. Cells expressing GFP include transduced lentivirus containing the sequence of interest. Since infections did not reaching 100% efficiency, the transduced cells were FACS sorted for GFP-positive cells.

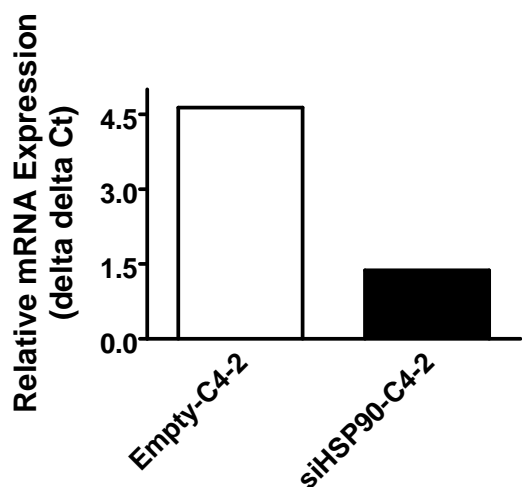


Fig. 3. Real-Time RT-PCR Analysis of Hsp90 mRNA expression in C4-2 Cells Transduced by Lentiviral siHsp90 Vector. Cells were harvested for at 90% confluency from a 6-well plate. RNA was isolated with Trizol® Reagent. cDNA synthesis and qPCR by Sybr Green were performed. The resultant graph is generated from one experiment done in triplicate. Expression of Hsp90 mRNA in siHSP90-C42 stable cells is knocked down by 3.4 fold relative to the empty vector infected control cells.

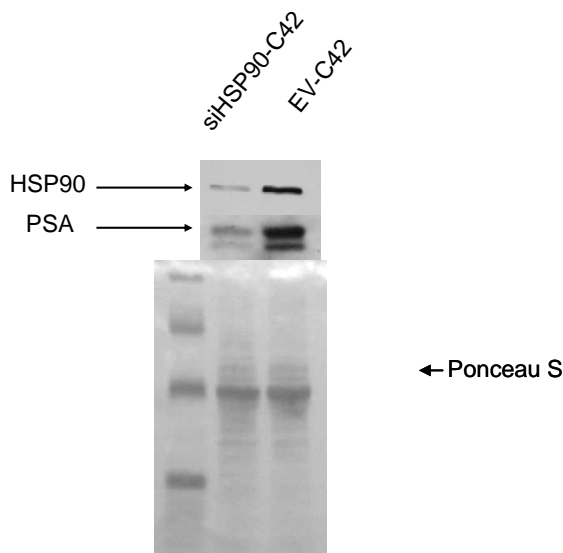


Fig. 4. Western Blot Analysis of Hsp90 Expression in C4-2 Cells Transduced by siHsp90 Lentiviral Vector. Cells were harvested in RIPA buffer containing protease inhibitors at ~90% confluency. Lysates were run on an SDS gel and transferred to membrane. Hsp90 was probed using anti-HSP90 $\alpha\beta$ (N-17). Empty vector infected C4-2 cells were used as a control in parallel.

We have tried to determine the endogenous AR localization in C4-2 cells infected with empty vector or with shHSP90 lenti-virus. However, immunohistochemical analysis revealed variable endogenous AR localization. Our results were not reproducible. Thus, we are doing troubleshooting now. Variability of our observations in AR localization could be due to the re-expression of HSP90 in the C4-2 cells infected with shHSP90. In fact, we showed that HSP90 expression was increased after several passages in C4-2 cells infected with shHSP90. Also, HSP90 expression can respond to stress, which could cause variability in our studies. While we are trying to resolve the technical difficulties, we explored alternative approaches to inhibit HSP90 activity. One mechanism of regulating HSP90 is its acetylation/deacetylation by HDAC6 (6-9). Thus, we decided to test the effect of HDAC6 on HSP90 acetylation and AR intracellular localization in C4-2 cells. One advantage is that HDAC6 knockdown did not affect cell viability (data not shown), which is consistent with the fact that HDAC6 knockout mice develop normally (10).

HDAC6 knockdown inhibits AR transcriptional activity.

To define the role of HDAC6 in the regulation of AR function, we established C4-2 cells stably expressing shRNA specific to HDAC6 using a lentiviral shRNA expression system. As shown in Figure 5A, HDAC6 protein level was knocked down approximately 50% as compared to the uninfected or control shRNA infected C4-2 cells. Moreover, protein level of HDAC10, which is most closely related to HDAC6 was not affected, suggesting that the shRNA effect was HDAC6 specific. We also observed that the protein levels of Hsp90 and AR were not affected by HDAC6 knockdown. HDAC6 knockdown inhibited both ligand-independent and androgen-induced PSA expression in C4-2 cells (Fig. 5A). Interestingly, DHT elevated PSA expression above basal levels in HDAC6 knockdown cells (Fig. 5A). This suggests that HDAC6 knockdown inhibits ligand-independent activation of AR while retaining androgen-responsiveness. Consistent with the results shown in Figure 5A, Real-time RT-PCR verified that HDAC6 knockdown inhibited PSA mRNA in C4-2 cells while retaining androgen-responsiveness (Fig. 5B). As expected, HDAC6 knockdown resulted in hyperacetylation of Hsp90 in C4-2 cells (Fig. 5C). However,

DHT treatment did not affect the level of acetylated Hsp90 (Fig. 5C). Expression of codon-switched HDAC6 expression vector, which is resistant to shHDAC6 treatment (Fig. 5D), restored PSA mRNA in HDAC6 knockdown C4-2 cells (Fig. 5E). This confirms that the shRNA effect is mediated through loss of HDAC6 and not by off target effects.

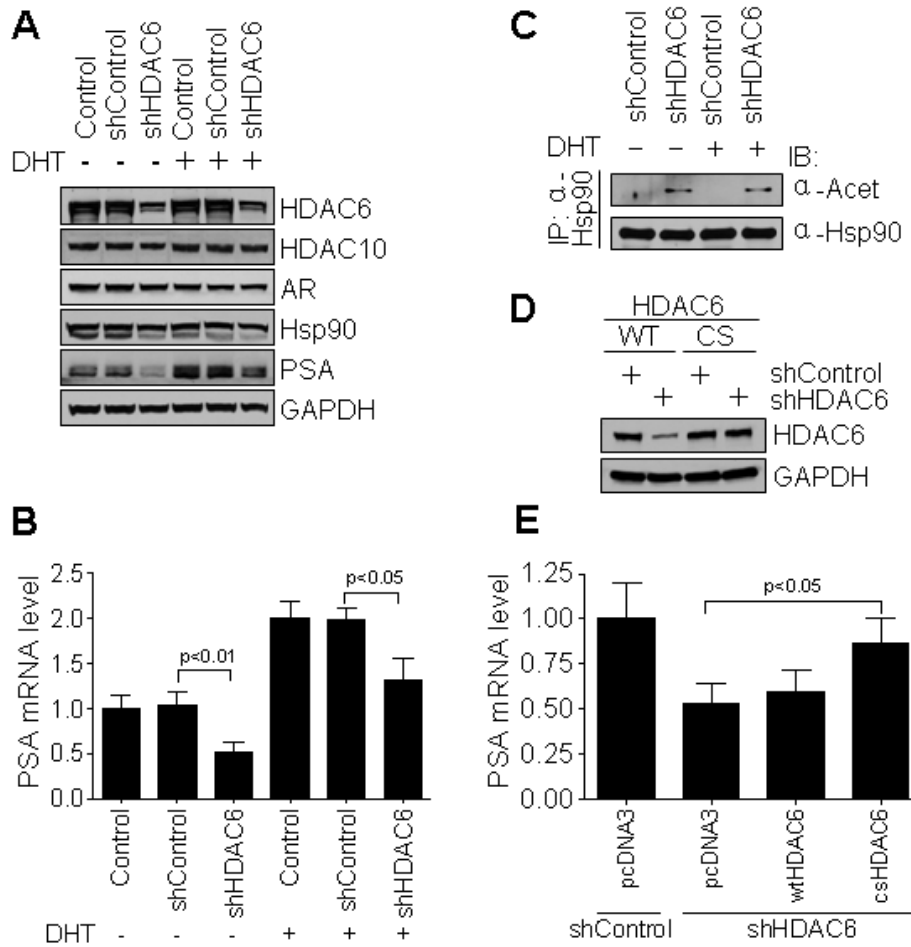


Fig.5. Effect of HDAC6 knockdown on endogenous AR expression and its transcriptional activity in prostate cancer cells. (A) C4-2 cells were infected with the lenti-virus encoding for shRNA to HDAC6 or Luciferase and stable cells were cultured in ligand-free conditions for 24 hours prior to treatment with (+) or without (-) 1nM DHT for additional 16 hours. Then the cell lysates were immunoblotted with anti-HDAC6, HDAC10, AR, Hsp90, PSA, or GAPDH antibody. (B) Total RNA isolated from C4-2 cells identically treated as Fig. 5A were used for real-time RT-PCR analysis to determine PSA mRNA level. (C) C4-2 cells with HDAC6 knockdown or shRNA control were cultured in conditions with or without DHT for 16 hours, and then Hsp90 was immunoprecipitated (IP) from the cell lysates and immunoblotted (IB) with either anti-Hsp90 or anti-acetylated lysine antibody. GAPDH served as loading control or internal control. (D) C4-2 cells were cotransfected with shHDAC6 or shControl vector and wild type (WT) or codon-switched (CS) HDAC6 expression vector and Flag-tagged HDAC6 expression was detected by anti-Flag antibody. (E) GFP positive cells were sorted and endogenous PSA mRNA was determined using CellsDirect One-Step qRT-PCR. Error bars represent \pm SD. A P-value <0.05 or <0.01 was generated using an unpaired *t*-test in GraphPad Prism (GraphPad Software, Inc.).

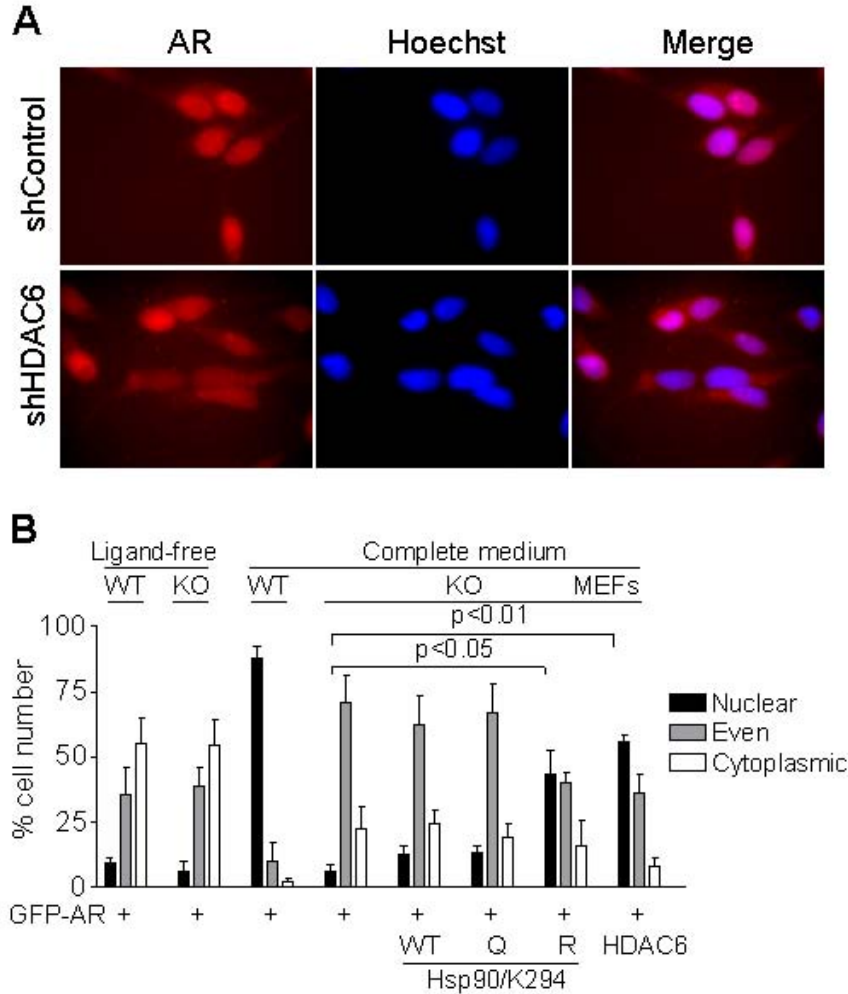


Fig. 6. Effects of HDAC6 knockdown or knockout on AR intracellular localization. (A) Control and HDAC6 knockdown C4-2 cells were cultured in ligand-free conditions for 24 hours and then the localization of AR was determined by immunostaining with an anti-AR antibody. Nuclei were stained with 1 μ g/ml Hoechst 33342. (B) Wild type and HDAC6 knockout MEFs were transfected with GFP-AR or HDAC6 knockout MEFs were co-transfected with GFP-AR and Hsp90 mutants or HDAC6 expression vector. Four hours after transfection, the cells were cultured in ligand-free or complete medium. Localization of GFP-AR was assessed by fluorescence microscopy after additional 16 hours culture. Results are the average of five experiments, where >100 cells were analyzed for each experiment. Error bars represent \pm SD. A P-value <0.05 or <0.01 was generated using an unpaired *t*-test in GraphPad Prism (GraphPad Software, Inc.).

HDAC6 regulates AR nuclear localization by deacetylation of Hsp90.

We determined the effect of HDAC6 knockdown on the intracellular localization of AR in C4-2 cells. HDAC6 stable knockdown and shRNA control C4-2 cells were cultured in ligand-free conditions for 24 hours and then cultured in ligand-free medium or medium containing 1 nM DHT for additional 16 hours. Localization of endogenous AR was determined by immunofluorescence experiments. As expected, in ligand-free conditions, endogenous AR is predominantly present in the nucleus of C4-2 cells expressing shRNA control (Fig. 6A). However, in HDAC6 knockdown C4-2 cells, AR is evenly distributed between cytoplasm and nucleus. Treatment with 1 nM DHT for 16 hours was sufficient to prevent HDAC6 knockdown-induced cytoplasmic localization of AR (Data not shown). Thus, the loss of HDAC6 prevents ligand-independent nuclear localization of endogenous AR in C4-2 cells. To better define the role of HDAC6 and explore its mechanism in regulating AR trafficking, we utilized wild type (WT) and HDAC6 knockout (KO) MEF cells to examine the GFP-AR intracellular localization in the absence or presence of ligand. As shown in Figure 6B, no difference in GFP-AR distribution was observed between WT and HDAC6 KO MEFs when the cells were cultured in ligand-free conditions. In both types of MEF transfected cells, ~35% of the cells showed even localization of GFP-AR and ~55% of the transfected cells showed cytoplasmic GFP-AR. In complete medium, which contains ligand, ~86% of transfected WT MEFs exhibited nuclear GFP-AR, whereas ~18% and ~70% of the transfected HDAC6 KO MEFs displayed nuclear and even GFP-AR localization, respectively. As a control, co-transfection of HDAC6 and GFP-AR plasmids in HDAC6 KO MEF cells increased the percentage of transfected cells with GFP-AR nuclear localization from ~18% to ~50% in complete medium. These results indicate that depletion of HDAC6 impaired AR nuclear localization in complete medium. We next investigated the mechanism by which HDAC6 inhibition suppresses nuclear localization of AR. Since HDAC6 deficiency caused hyperacetylation of Hsp90 in C4-2 cells (Fig. 5C) and MEF cells (10), and the acetylation state of Hsp90 K294 is critical for its chaperone function (11), we used Hsp90 K294 mutants to determine whether acetylation/deacetylation of Hsp90 is responsible for HDAC6 mediated nuclear localization of AR. Expression of a wild-type or an acetylation mimic Hsp90 (Hsp90K294Q) slightly increased the percentage of nuclear GFP-AR, while a deacetylation mimic Hsp90 mutant (Hsp90K294R) markedly restored nuclear localization of GFP-AR, to the extent similar to the restoration by HDAC6 re-expression. These observations argue that defect of GFP-AR nuclear translocation caused by HDAC6 deficiency is mediated mainly through acetylation of Hsp90.

Task 2: Test the hypothesis that HSP90 modulates the sensitivity of AR-positive xenograft tumors to androgen ablation (months 10-30)

We were not able to test the effect of HSP90 knockdown on xenograft tumor growth because of phenotype changes of C4-2 cells expressing shHSP90. Specifically, HSP90 levels rebounded after a few passages in C4-2 sublines with shHSP90 expression. One alternative is to conduct the xenograft tumor studies using C4-2 cells with HDAC6 knockdown instead of with HSP90 knockdown. This is not surprising because HSP90 is essential for cell survival. Since HDAC6 regulates AR activity via HSP90 acetylation/deacetylation, we decided to test the effect of inhibiting HSP90 via blocking its deacetylation on C4-2 xenograft tumor growth.

HDAC6 knockdown inhibits C4-2 xenograft tumor development and growth accompanied by decreased PSA levels in xenograft tissues. To determine whether HDAC6 silencing could affect AR transcriptional activity and inhibit prostate cancer growth *in vivo*, we established human prostate xenograft tumors in nude mice (Fig. 7A). Both C4-2/shControl and C4-2/shHDAC6 cells generated tumors in six out of six (100%) intact mice (Fig. 7C). HDAC6 knockdown inhibited tumor growth in intact mice 6 weeks after injection compared with control (Fig. 7B). The means of the tumor volumes of C4-2/shControl ($2.12 \pm 0.33\text{cm}^3$) and C4-2/shHDAC6 ($0.96 \pm 0.26\text{cm}^3$) were significantly different ($p < 0.05$), suggesting that HDAC6 gene function is important to the growth of androgen-independent prostate xenograft tumors. The inhibition of xenograft tumor growth corresponded with a marked decrease in PSA expression levels in tumor tissues without affecting AR expression levels (Fig. 7D). This indicated that HDAC6 knockdown inhibited AR transcriptional activity even in intact male mice. No tumor was formed in castrated nude mice injected with C4-2/shHDAC6 cells while C4-2/shControl cells formed tumors in two out of six (33%) castrated mice (Fig. 7C). These findings argue that HDAC6 knockdown inhibited the establishment of castration-resistant prostate cancer *in vivo*.

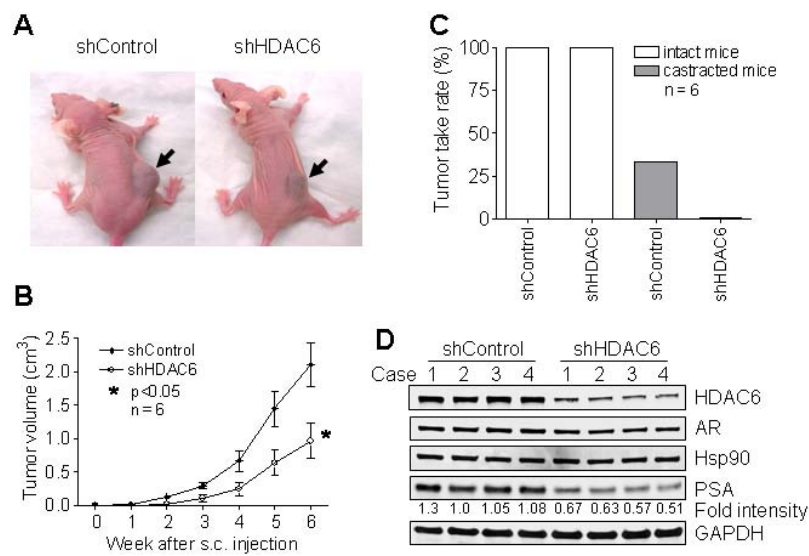


Fig. 7. HDAC6 knockdown inhibits C4-2 xenograft tumor growth. (A) Xenograft tumor formation in intact mice, indicated by an arrow. (B) Tumor growth is shown as a function of tumor volume at indicated days post s.c. injection. Error bars represent \pm SEM. * A P-value < 0.05 was generated using an unpaired *t*-test in GraphPad Prism (GraphPad Software, Inc.). (C) Tumor take rate was calculated 12 weeks after s.c. injection. (D) Tissue lysates of xenografts collected from intact mice were immunoblotted with anti-HDAC6, anti-Hsp90, anti-AR and anti-PSA antibodies. GAPDH served as loading control.

Task 3: Determine the efficacy of HSP90 inhibitor in the inhibition of the prostate cancer progression to androgen-independence in LNCaP xenograft tumor model (months 20-36):

We started Task 3 in the third year funding period. A major problem encountered was the low LNCaP tumor take rate in nude mice (<10%), which was much lower than what we had previously for LNCaP (~60-70%) (12). To overcome this difficulty, we obtained another AR-positive androgen-sensitive prostate cancer cell line, LuCaP35, from Dr. Robert Vessella at University of Washington (13). A major advantage of the LuCaP35 model is that it has wild-type AR, since most of prostate cancers, including castration-resistant prostate cancer, have wild-type AR (14-16). We have established LuCaP35 xenograft tumors. The tumor take rate of LuCaP35 was about 60-70% in nude mice, which should make the proposed experiment feasible. We anticipate that the experiment will be completed in July, 2010.

Key Research Accomplishments

1. HSP90 acetylation is required for ligand-independent AR nuclear localization in C4-2 castration-resistant prostate cancer cells.
2. HDAC6 regulation of ligand-independent AR nuclear localization in C4-2 cells is mediated through HSP90 acetylation/deacetylation.
3. HDAC6 knockdown by shRNA inhibited establishment of C4-2 xenograft tumors in castrated nude mice.

Reportable Outcomes

1. Junkui Ai, Yujuan Wang, Javid A. Dar, June Liu, Lingqi Liu, Joel B. Nelson, Zhou Wang: HDAC6 Regulates Androgen Receptor Hypersensitivity and Nuclear Localization via Modulating Hsp90 Acetylation in Castration-resistant Prostate Cancer, *Molecular Endocrinology* 2009, 23: 1963-1972.

Conclusion

HSP90 acetylation/deacetylation, which is modulated by HDAC6, is a critical step involved in ligand-independent nuclear localization of AR and the development of castration resistance of prostate cancer cells.

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Appendices

A manuscript is attached.

1. Junkui Ai, Yajuan Wang, Javid A. Dar, June Liu, Lingqi Liu, Joel B. Nelson, Zhou Wang: HDAC6 Regulates Androgen Receptor Hypersensitivity and Nuclear Localization via Modulating Hsp90 Acetylation in Castration-resistant Prostate Cancer, *Molecular Endocrinology* 2009, 23: 1963-1972.

HDAC6 Regulates Androgen Receptor Hypersensitivity and Nuclear Localization via Modulating Hsp90 Acetylation in Castration-Resistant Prostate Cancer

Junkui Ai, Yujuan Wang, Javid A. Dar, June Liu, Lingqi Liu, Joel B. Nelson, and Zhou Wang

Department of Urology (J.A., Y.W., J.A.D., J.L., L.L., J.B.N., Z.W.), Department of Pathology (Y.W.), and Department of Pharmacology and Chemical Biology (Z.W.), University of Pittsburgh School of Medicine, University of Pittsburgh Cancer Institute (J.B.N., Z.W.), Pittsburgh, Pennsylvania 15232

The development of castration-resistant prostate cancer (PCa) requires that under castration conditions, the androgen receptor (AR) remains active and thus nuclear. Heat shock protein 90 (Hsp90) plays a key role in androgen-induced and -independent nuclear localization and activation of AR. Histone deacetylase 6 (HDAC6) is implicated, but has not been proven, in regulating AR activity via modulating Hsp90 acetylation. Here, we report that knockdown of HDAC6 in C4-2 cells using short hairpin RNA impaired ligand-independent nuclear localization of endogenous AR and inhibited PSA expression and cell growth in the absence or presence of dihydrotestosterone (DHT). The dose-response curve of DHT-stimulated C4-2 colony formation was shifted by shHDAC6 such that approximately 10-fold higher concentration of DHT is required, indicating a requirement for HDAC6 in AR hypersensitivity. HDAC6 knockdown also inhibited C4-2 xenograft tumor establishment in castrated, but not in testes-intact, nude mice. Studies using HDAC6-deficient mouse embryonic fibroblasts cells showed that inhibition of AR nuclear localization by HDAC6 knockdown can be largely alleviated by expressing a deacetylation mimic Hsp90 mutant. Taken together, our studies suggest that HDAC6 regulates AR hypersensitivity and nuclear localization, mainly via modulating HSP90 acetylation. Targeting HDAC6 alone or in combination with other therapeutic approaches is a promising new strategy for prevention and/or treatment of castration-resistant PCa. (*Molecular Endocrinology* 23: 1963–1972, 2009)

The androgen receptor (AR) mediates the effect of androgens on both prostate development and prostate cancer (PCa) initiation and progression (1–4). PCa is initially androgen dependent, and so most patients respond favorably to androgen ablation, the standard form of treatment for locally advanced or metastatic disease. However, PCa almost always progresses and becomes resistant to this treatment (5–8). The current preferred term describing the growth of PCa under androgen-depleted conditions is “castration-resistant,” which is thought to be more accurate than “androgen-independent,” “hormone-

independent,” “androgen-refractory,” “hormone-refractory,” and “androgen-insensitive,” etc. Castration resistance requires active AR via the overexpression, mutation, and/or posttranslational modification of AR and/or its coactivators (2, 6, 7, 9–14). AR hypersensitization to ligand may also activate AR in castration-resistant PCa cells (15), but the mechanisms underlying AR hypersensitivity remain unclear.

AR is a member of the nuclear steroid receptor superfamily (2). Without ligand, AR is inactive and resides predominantly in the cytoplasm bound to chaperones,

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Abbreviations: 17-AAG, 17-allylamino-17-demethoxygeldanamycin; AR, androgen receptor; DHT, dihydrotestosterone; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; GFP, green fluorescent protein; GR, glucocorticoid receptor; HDAC6, histone deacetylase 6; Hsp90, heat shock protein 90; MEF, mouse embryonic fibroblast; NaBut, sodium butyrate; PCa, prostate cancer; PSA, prostate-specific antigen; qRT-PCR, quantitative RT-PCR; shRNA, short hairpin RNA; TSA, trichostatin A.

including heat shock proteins (Hsp) (16–19). Upon ligand binding, AR undergoes conformational changes and nuclear translocation, resulting in interactions with transcriptional cofactors. AR can then induce expression of specific genes involved in an array of cellular activities, including homeostasis, growth, reproduction, development, and metabolism (20–22). Steroid hormone receptors require additional cellular machinery to achieve the appropriate conformation for binding ligand (23). For example, heat shock protein 90 (Hsp90) plays a central role in the formation of a multichaperone complex essential for stabilizing steroid receptors in a conformation receptive to ligand (24, 25). Hsp90 regulates the client protein half-life by forming conformation-dependent higher order chaperone complexes (26). Hsp90 inhibition prevents the ligand-dependent nuclear translocation of AR, suggesting a role for Hsp90 in the nuclear import of AR (27). Our previous study suggested that ligand-independent AR nuclear localization in castration-resistant PCa cells also requires Hsp90 (28).

Histone deacetylase 6 (HDAC6) reversibly acetylates Hsp90, modulating Hsp90 regulation of nuclear receptors, such as the glucocorticoid receptor (GR) (29–33). Inactivation of HDAC6 results in the accumulation of acetylated Hsp90, which no longer forms a stable complex with GR, leading to defective GR ligand binding, nuclear translocation, and transactivation (29, 30). These findings strongly implicate HDAC6-mediated acetylation/deacetylation of Hsp90 as a potential mechanism regulating steroid hormone signaling. Because AR forms chaperone complexes with Hsp90 (34) and recently it was reported that HDAC6 is required for stabilization of AR protein (35), it is thought, but has not been demonstrated, that HDAC6 also regulates AR via modulating Hsp90 acetylation. It is also not clear whether HDAC6 is involved in AR activation in castration-resistant PCa.

Here we investigated the role of HDAC6 in AR intracellular trafficking and function in PCa cells. We demonstrated that inactivation of HDAC6 inhibited AR nuclear localization and subsequent transactivation in PCa and mouse embryonic fibroblast (MEF) cells. Reexpressing HDAC6 or a deacetylation-mimic Hsp90 mutant alleviated the inhibition. Interestingly, HDAC6 knockdown shifted the dose response of C4-2 growth to dihydrotestosterone (DHT) such that approximately 10-fold more DHT was required. Furthermore, HDAC6 knockdown also inhibited the establishment of C4-2 xenograft tumors in castrated, but not testis-intact, nude mice. These findings together provide evidence for an important role for HDAC6 in AR hypersensitivity and nuclear localization in castration-resistant PCa cells.

Results

Trichostatin A (TSA) inhibited the ligand-independent nuclear localization of green fluorescent protein tagged AR (GFP-AR) in C4-2 cells

To examine whether ligand-independent nuclear localization of AR requires HDAC6 deacetylase activity, we transfected castration-resistant PCa C4-2 cells with GFP-AR and then treated cells with the pan-HDAC inhibitor, TSA, or with sodium butyrate (NaBut), which inhibits all HDACs except HDAC6 (36). GFP tagging does not affect the function, subcellular localization, and stability of the AR protein (Refs. 21, 37, 38 and data not shown). Without ligand, GFP-AR in C4-2 cells was predominately nuclear, in contrast to the predominant cytoplasmic localization of GFP-AR in androgen-sensitive LNCaP cells. As a control, we showed that GFP alone was evenly distributed in both LNCaP and C4-2 cells. In C4-2 cells, TSA treatment produced a shift of GFP-AR to the cytoplasm whereas NaBut treatment did not (Fig. 1, A and B). As expected, the localization of GFP control was insensitive to TSA or NaBut treatment. Western blot showed that TSA slightly enhanced GFP-AR expression (Fig. 1C), indicating that the redistribution of GFP-AR was not caused by selective degradation of nuclear GFP-AR. Thus, inhibition of HDAC6 deacetylase activity by TSA appears to prevent the ligand-independent nuclear localization of GFP-AR in castration-resistant C4-2 cells.

TSA reduced endogenous AR in PCa cells

To determine the effect of TSA on endogenous AR expression and transcriptional activity, we treated C4-2 and LNCaP cells with ethanol, TSA, or NaBut in the absence or presence of 1 nM DHT for 16 h. In both cell lines, TSA decreased AR protein levels, as well as inhibited basal and androgen-induced prostate-specific antigen (PSA) expression at the protein and mRNA level (Fig. 2, A and C). TSA did not affect HDAC6 or Hsp90 protein levels. Due to the opposite effects of TSA on exogenous and endogenous AR protein levels (Fig. 1C), we speculated that TSA might regulate endogenous AR expression transcriptionally. Quantitative RT-PCR verified that TSA decreased endogenous AR mRNA levels in C4-2 and LNCaP cells in the absence or presence of DHT (Fig. 2B). Although inhibition of HDAC6 by TSA may play a significant role in chemical ablation of endogenous AR in both androgen-sensitive and castration-resistant PCa cells, the inhibition of endogenous AR expression by TSA may be mediated through other pathways as well.

HDAC6 knockdown inhibited AR transcriptional activity

To define the role of HDAC6 in AR regulation, we established C4-2 cells stably expressing HDAC6 short

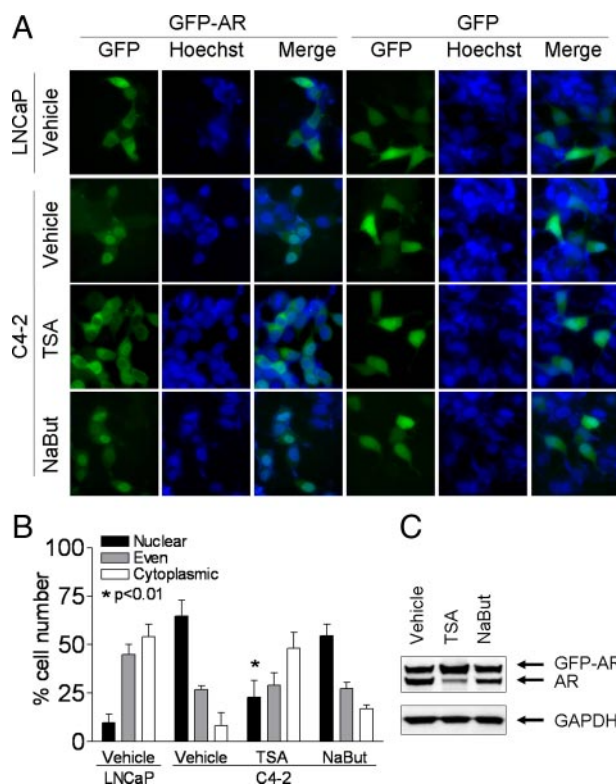


FIG. 1. Effect of the HDAC6 inhibitor TSA on GFP-AR localization and expression in castration-resistant PCa cells. Castration-resistant C4-2 or androgen-sensitive LNCaP cells were transfected with GFP-AR and 4 h later were treated with ethanol (vehicle), 1 μ M TSA, or 1 mM NaBut in ligand-free conditions. A, Localization of GFP-AR in C4-2 or LNCaP cells was assessed by fluorescence microscopy 16 h after treatment. B, Results are the average of five experiments, in which more than 100 cells were analyzed for each experiment. Error bars represent \pm SD. *, A *P* value < 0.05 was generated using an unpaired *t* test in GraphPad Prism. C, Effect of TSA on GFP-AR protein levels in C4-2 cells. Whole-cell lysates of C4-2 cells with identical treatments as in panel A were subjected to SDS-PAGE, and GFP-AR levels were determined by Western blot analysis.

hairpin RNA (shRNA) using a lentiviral shRNA expression system. shRNA knocked down HDAC6 protein levels by approximately 50% as compared with controls, but did not knock down the closely related HDAC10, suggesting a specific effect (Fig. 3A). Whereas TSA depleted endogenous AR, HDAC6 knockdown did not affect Hsp90 or AR levels; similar to what was reported for GR and PR (24, 39). HDAC6 knockdown did inhibit both ligand-independent and androgen-induced PSA protein expression; however, unlike in TSA-treated cells, DHT elevated PSA expression above basal levels in these cells (Fig. 3A). This suggests that HDAC6 knockdown inhibits ligand-independent activation of AR but not androgen responsiveness. These findings also suggest that TSA and HDAC6 knockdown do not interfere with AR function through identical mechanisms. Real-time RT-PCR verified that HDAC6 knockdown inhibited PSA mRNA expression while retaining androgen responsiveness (Fig. 3B). HDAC6 effect on PSA mRNA level is likely at the

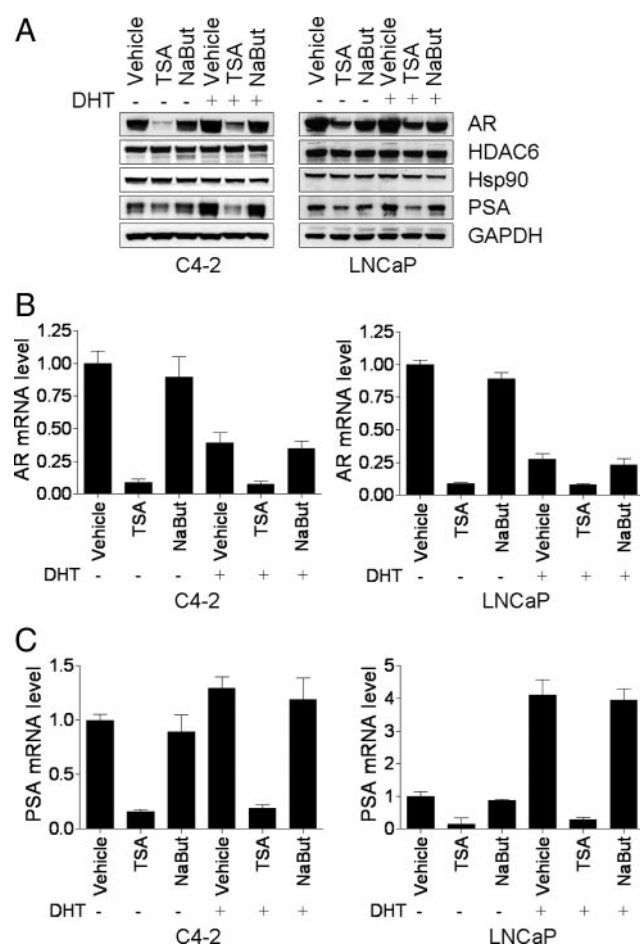
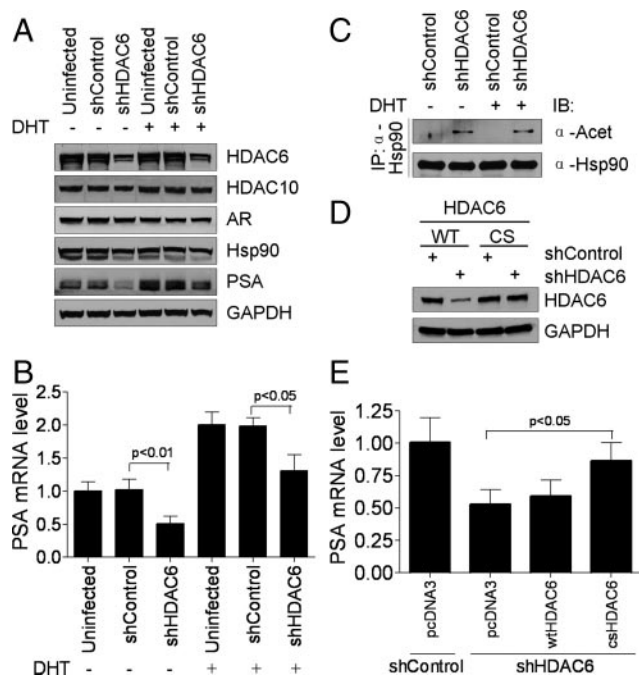


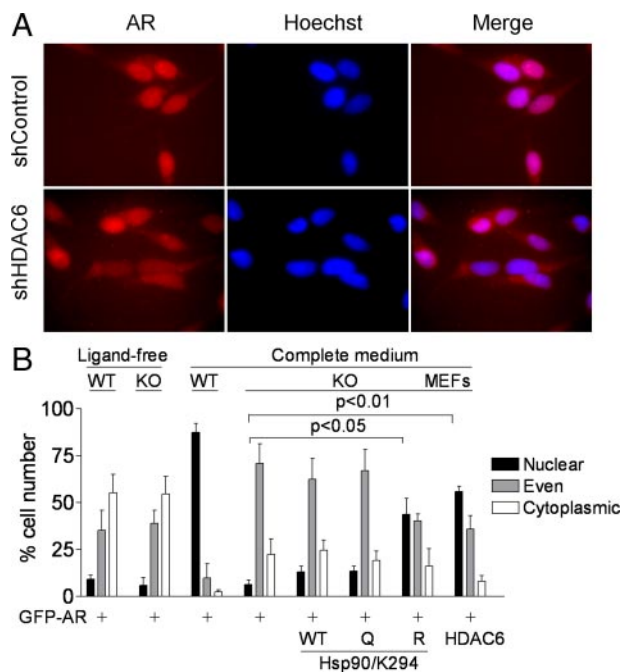
FIG. 2. Effect of HDAC6 inhibitor TSA on endogenous AR expression and transcriptional activity in PCa cells. A, C4-2 and LNCaP cells were cultured in ligand-free conditions for 24 h before treatment with ethanol (vehicle), 1 μ M TSA, or 1 mM NaBut in the absence (–) or presence (+) of 1 nM DHT. The cell lysates were collected 16 h after treatment and were immunoblotted with anti-AR, HDAC6, Hsp90, or PSA antibody. GAPDH was used as loading control. Total RNA from cells with identical treatment as panel A were used for real-time RT-PCR analysis to determine AR (B) and PSA (C) mRNA levels in C4-2 and LNCaP cells. Error bars represent \pm SD. GAPDH was used to normalize the quantitative real-time PCR results.

transcription level because PSA mRNA stability is not altered by shHDAC6 (Data not shown). To characterize HDAC6 activity in C4-2 cells, we tested whether HDAC6 knockdown can result in Hsp90 hyperacetylation in the absence or presence of DHT. The results in Fig. 3C indicate that HDAC6 is functional and can deacetylate Hsp90 and that Hsp90 acetylation/deacetylation is independent of androgens in C4-2 cells. To further validate that HDAC6 is involved in regulation of AR signaling in C4-2 cells, we tested whether the expression of codon-switched HDAC6, which resists the knockdown by shRNA-HDAC6 (Fig. 3D), can restore the expression of AR-target PSA in C4-2/shHDAC6 cells (Fig. 3E). The restoration of the PSA mRNA level (Fig. 3E) argues that the shHDAC6 effect on AR signaling is mediated specifically through loss of HDAC6.



HDAC6 regulated AR nuclear localization by deacetylation of Hsp90

Given the association between AR function and its intracellular localization, we next determined the effect of HDAC6 knockdown on AR localization. We cultured C4-2/shHDAC6 and C4-2/shControl cells in ligand-free conditions for 24 h and then in the presence or absence of 1 nM DHT for 16 h. We determined endogenous AR localization by immunofluorescence. As expected, without DHT, AR predominantly resided in the nucleus of C4-2/shControl cells (Fig. 4A). However, in C4-2/shHDAC6 cells, AR was evenly distributed between the cytoplasm and nucleus. DHT treatment of HDAC6-knockdown cells blocked cytoplasmic AR localization (data not shown). Next, we used wild-type and HDAC6-knockout



MEF cells to examine GFP-AR localization. As shown in Fig. 4B, wild-type and HDAC6-knockout MEFs cultured in ligand-free conditions displayed a similar GFP-AR distribution: approximately 35% of cells showed even localization and about 55% showed cytoplasmic localization. Differences between the two MEFs emerged in the presence of ligand. In complete medium, about 86% of wild-type MEFs exhibited nuclear GFP-AR, whereas we observed nuclear GFP-AR in only approximately 18% of HDAC6-knockout MEFs with even localization in about 70%. Cotransfection of HDAC6 and GFP-AR plasmids in HDAC6-knockout MEF cells in complete medium increased the percentage of cells with GFP-AR nuclear localization from approximately 18% to about 50%.

We next investigated the mechanism by which HDAC6 inhibition suppresses AR nuclear localization. Because HDAC6 deficiency caused hyperacetylation of Hsp90 in C4-2 cells (Fig. 3C) and MEF cells (39), and the acetylation state of Hsp90 K294 is critical for its chaperone function (40), we used Hsp90 K294 mutants to

determine whether acetylation/deacetylation of Hsp90 is responsible for HDAC6-mediated nuclear localization of AR. Expression of a wild-type or an acetylation mimic Hsp90 (Hsp90K294Q) slightly increased the percentage of nuclear GFP-AR, whereas a deacetylation mimic Hsp90 mutant (Hsp90K294R) markedly restored GFP-AR nuclear localization to levels seen with HDAC6 reexpression (Fig. 4B). These observations argue that defect of GFP-AR nuclear translocation caused by HDAC6 deficiency is mediated mainly through acetylation of Hsp90.

HDAC6 knockdown shifted androgen dose response toward higher DHT concentrations in C4-2 clonogenicity assay

Given that androgens mediate cell proliferation and survival in PCa cells through AR (41), we evaluated the effect of HDAC6 knockdown on PCa cell proliferation. We cultured C4-2/shHDAC6 and C4-2/shControl cells in complete or ligand-free media and counted cells at d 2, d 4, and d 6. Regardless of ligand, HDAC6 knockdown only slightly inhibited C4-2 cell proliferation at d 6 (Fig. 5, A and B; $P < 0.01$). No cell death was observed in C4-2/shHDAC6 cells (data not shown). Taken together, these data argue that HDAC6 inhibition causes little or no cytotoxicity. We also measured C4-2 colony formation at various androgen concentrations. C4-2 cells exhibited bell-shaped growth response to increasing DHT concentrations (Fig. 5, C and D), which is similar to the dose response of androgen-induced LNCaP growth (42). C4-2/shHDAC6 cells developed visibly smaller colonies than C4-2/shControl cells (data not shown). Moreover, HDAC6 knockdown caused a rightward shift in the dose response to DHT: whereas 10^{-10} M DHT stimulated the maximum colony formation in the control group, HDAC6-knockdown cells required 10^{-9} M DHT (Fig. 5, C and D).

HDAC6 knockdown inhibited C4-2 xenograft tumor development, growth, and PSA levels

To evaluate the effect of HDAC6 *in vivo*, we established human prostate xenograft tumors in nude mice (Fig. 6A). Both C4-2/shControl and C4-2/shHDAC6 cells generated tumors in six of six (100%) intact mice (Fig. 6C). However, 6 wk after injection (Fig. 6B) the mean volume of C4-2/shControl tumors in intact mice (2.12 ± 0.33 cm³) was statistically significantly greater than that of C4-2/shHDAC6 tumors (0.96 ± 0.26 cm³; $P < 0.05$), supporting a role for HDAC6 in androgen-dependent prostate xenograft tumor growth. C4-2/shHDAC6 xenograft tumors exhibited a marked decrease in PSA expression while having little or no change in AR protein levels. (Fig. 6D), indicating that HDAC6 knockdown inhibited

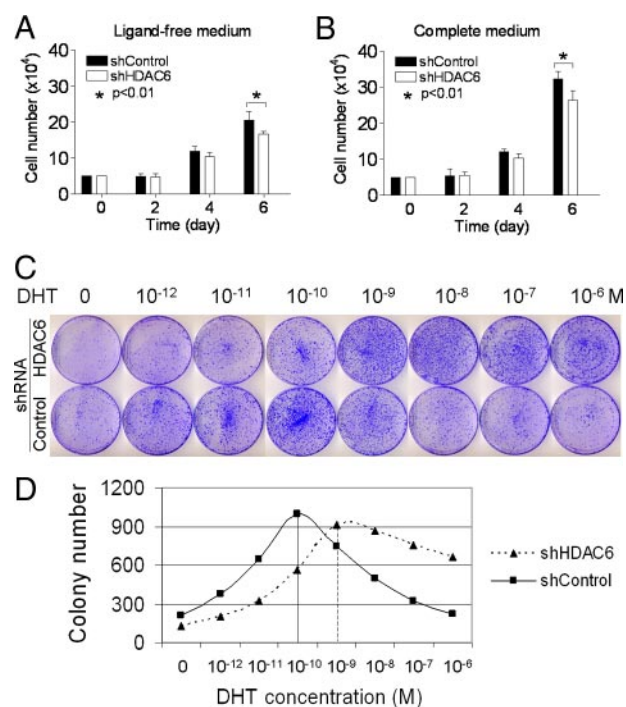


FIG. 5. Effect of HDAC6 knockdown on PCa cell growth. Control and HDAC6-knockdown C4-2 cells were cultured in ligand-free (A) or complete (B) medium with an initial 50,000 cells per well on six-well plates in triplicate. The cell number was counted at the indicated time points. The results presented here are representative of three independent experiments. Error bars represent \pm SD. *, A P value < 0.01 was generated using an unpaired t test in GraphPad Prism. C, Control and HDAC6-knockdown C4-2 cells were seeded at 10,000 cells per 10-cm dish in triplicate. After 2 wk in T-medium without or with the indicated concentrations of DHT, cell colonies were stained with crystal violet and colony numbers counted. The colony images are representative of three independent experiments. D, Quantitative analysis of colony formation assay. The colony numbers represent means.

AR transcriptional activity, even in intact male mice. No tumor was formed in castrated nude mice injected with C4-2/shHDAC6 cells whereas C4-2/shControl cells formed tumors in two of six (33%) castrated mice (Fig. 6C), arguing that HDAC6 knockdown inhibits the establishment of castration-resistant PCa *in vivo*.

Discussion

Elucidating the mechanisms responsible for AR activation in castration-resistant PCa cells has significant implications in the prevention and treatment of advanced disease. HDAC6 is implicated, but has not been proven, to play a key role in regulating AR activity in castration-resistant PCa cells. Using the castration-resistant PCa cell line C4-2 as a model, we showed that HDAC6 is required for AR hypersensitivity, AR nuclear localization, and castration resistance. Further analysis suggests that HDAC6 regulates AR via modulating Hsp90 acetylation. Our

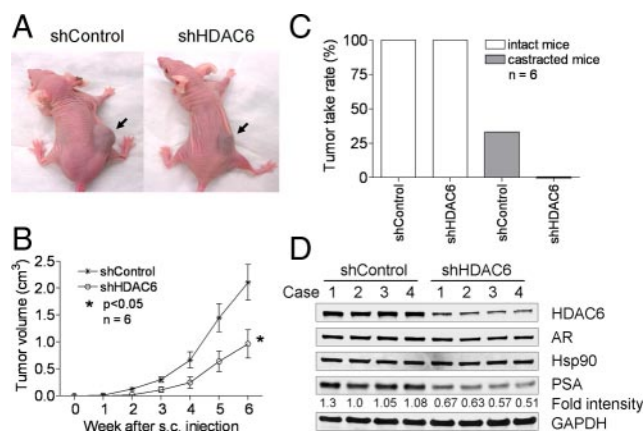


FIG. 6. HDAC6 knockdown inhibits C4-2 xenograft tumor growth. **A**, Xenograft tumor formation in intact mice, indicated by an arrow. **B**, Tumor growth is shown as a function of tumor volume at indicated days after s.c. injection. Error bars represent \pm SEM. *, P value < 0.05 was generated using an unpaired t test in GraphPad Prism. **C**, Tumor take rate was calculated 12 wk after s.c. injection. **D**, Tissue lysates of xenografts collected from intact mice were immunoblotted with anti-HDAC6, anti-Hsp90, anti-AR, and anti-PSA antibodies. GAPDH served as loading control.

findings argue that HDAC6 is a promising target for castration-resistant PCa treatment.

HDACs can employ different mechanisms to regulate AR activity (43–45). Results from this and other studies showed that TSA decreases endogenous AR expression at both mRNA and protein levels (44, 45). However, there are conflicting reports that TSA enhances endogenous AR mRNA in LNCaP cells (46, 47), and the reason for this discrepancy is unclear. In our experiments, NaBut, unlike TSA, did not alter AR localization or expression. Nevertheless, it has been reported that prolonged or increased NaBut treatment inhibited AR expression and activity (43). A recent study showed that genetic knockdown of HDAC1 or HDAC3 suppressed the expression of AR-regulated genes, which is consistent with findings using HDAC inhibitors (43). Our studies showed that HDAC inhibitors can also affect GFP-AR nuclear localization via inhibiting HDAC6. Blocking any of the steps required for AR activation could inhibit the growth of castration-resistant PCa.

HDAC6 appears to play a critical role in regulating AR sensitivity to androgens, particularly the hypersensitivity in castration-resistant PCa. In C4-2 cells, HDAC6 knockdown inhibited PSA mRNA expression in the absence or presence of DHT. Similarly, HDAC6 knockdown reduced cell proliferation in both ligand-free and complete medium. Interestingly, DHT still induced PSA mRNA in C4-2/shHDAC6 cells, indicating that AR remains androgen responsive. Recent studies suggested that AR activation, as reflected by PSA expression, in castration-resistant PCa is not truly androgen independent (6). Hypersensitization of AR to castration level of androgens in PCa is thought to

be a major mechanism leading to castration resistance (15). The inhibition of PSA expression in C4-2/shHDAC6 cells cultured in charcoal-stripped medium, as well as the shift in the dose-response curve seen with colony assays, indicates that HDAC6 contributes to AR hypersensitivity. Moreover, we found that C4-2/shHDAC6 cells produced tumors in testes-intact, but not castrated, nude mice, further supporting an important role for HDAC6 in AR hypersensitivity and subsequent castration-resistant PCa growth.

The inhibition of AR hypersensitivity by HDAC6 knockdown is associated with the inhibition of AR nuclear localization in ligand-free medium. Two possible explanations exist for this observation. One possibility is that AR nuclear localization does not require androgen, only requires functional HDAC6, and the nuclear localization confers AR hypersensitivity. Alternatively, AR nuclear localization may be a result of AR hypersensitivity, which is inhibited by HDAC6 knockdown. Further studies are needed to clarify which is true. However, our results using MEF cells suggest that the effect of HDAC6 knockdown on AR subcellular localization is ligand dependent. In ligand-free medium, wild-type and HDAC6-knockout MEF cells display the same GFP-AR localization, indicating that HDAC6 does not modulate AR localization under this condition. Presumably, AR hypersensitivity exists only in castration-resistant PCa cells, but not in other types of cells such as MEFs. Thus, GFP-AR in MEFs (Fig. 4B) and castration-resistant C4-2 (Fig. 3B) are expected to have different sensitivities to DHT, with GFP-AR being active under androgen-depleted condition in C4-2 but not in MEF cells. Under androgen-free culture condition, HDAC6 knockdown affected the subcellular localization of active AR in C4-2 (Fig. 3B), but not inactive AR in MEF cells (Fig. 4B). When MEF cells are cultured in complete medium, HDAC6 knockout prevented GFP-AR nuclear localization, which is presumably induced by low levels of androgens present in the complete medium. Our results suggest that AR nuclear localization is secondary to its response to androgens, which is regulated by HDAC6.

Furthermore, our results suggest that HDAC6 regulates AR through acetylation/deacetylation of Hsp90, consistent with how HDAC6 regulates GR (29, 31, 47). This suggests that HDAC6 has potential to affect the activity of many other members in the nuclear receptor superfamily via regulating Hsp90 acetylation status. It has been identified that acetylation/deacetylation of Hsp90 K294 is critical for its chaperone function (40). Our studies showed that ectopic expression of a deacetylation mimic Hsp90 mutant (Hsp90 K294R) restored nuclear localization of GFP-AR in HDAC6-knockout MEF cells

in complete medium, and the extent of the restoration was similar to that induced by HDAC6 ectopic expression, suggesting a role for Hsp90 deacetylation in HDAC6-mediated AR regulation.

Inhibiting HDAC6 appears to have different effects from that of Hsp90 inhibitors on AR in PCa cells, although HDAC6 regulation of AR is mediated through Hsp90. Our previous studies showed that the Hsp90 inhibitor 17-allylamino-17-demethoxygeldanamycin (17-AAG) prevented androgen-independent AR nuclear localization in C4-2 cells (28). However, 17-AAG also caused AR degradation and significant cytotoxicity (48, 49). In contrast, whereas HDAC6 knockdown inhibited castration-resistant AR nuclear localization in C4-2 cells, it did not cause AR degradation and cell death. The lack of cytotoxicity upon HDAC6 knockdown is consistent with the lack of a detectable phenotype in HDAC6-knockout mice (39). The above observations suggest that 17-AAG and HDAC6 knockdown inhibit Hsp90 by different mechanisms. This raises an interesting possibility of using a combination of low-dose Hsp90 and HDAC6 inhibitors to kill PCa cells.

In summary, our studies provided experimental evidence that HDAC6 is required for the AR hypersensitivity essential in castration-resistant growth of PCa cells, and that HDAC6 regulation of AR is mediated mainly through Hsp90 acetylation/deacetylation. Because HDAC6 inhibition does not seem to affect cell survival and animal development (39), specific inhibition of HDAC6 enzymatic activity is likely to have fewer side effects. Taken together, targeting HDAC6 alone or in combination with other therapeutic agents is a promising new approach for prevention and treatment of castration-resistant PCa.

Materials and Methods

Reagents and plasmids

Trichostatin A (TSA), NaBut, and dihydrotestosterone (DHT) were purchased from Sigma (St. Louis, MO). Hoechst 33342 solution was purchased from Invitrogen (Carlsbad, CA). Anti-HDAC6 (H-300, L-18), HDAC3 (H-99), Hsp90 α/β (N-17), PSA (C-19), glyceraldehyde-3-phosphate dehydrogenase (GAPDH) (FL-335), AR (N-20) antibodies, and Protein A/G PLUS-Agarose were purchased from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA). Acetylated-lysine antibody was obtained from Cell Signaling Technology (Beverly, MA). Anti-HDAC10 antibody was obtained from BioVision, Inc. (Mountain View, CA). The GFP-AR construct was generated as described previously (50). Flag-tagged HDAC6 expression vector (51) was kindly provided by Dr. Eric M. Verdin (Gladstone Institute of Virology and Immunology, University of California, San Francisco). The codon-switched HDAC6 expression vector was constructed using the PCR-based site mutation method; eight nucleotides in the shRNA-targeting region were mutated without changing amino acids. The codon-switched sequence is as follows (mutated nucleotides are *underlined*): 5'-

GCATGGACTTAAATTTAGAA-3'. Flag-tagged wild-type Hsp90 α and Hsp90 α mutants K294R and K294Q were constructed as described previously (40) and were kindly provided by Dr. Len Neckers (National Cancer Institute, Bethesda, MD).

Cell culture and transfection

Androgen-sensitive PCa LNCaP cells were obtained from American Type Culture Collection (Manassas, VA) and maintained in RPMI 1640 medium supplemented with 10% fetal bovine serum, 1% glutamine, 100 U/ml penicillin, and 100 μ g/ml streptomycin (Invitrogen). The castration-resistant PCa C4-2 cell line, which was derived from LNCaP cells, was purchased from UroCor (Oklahoma City, OK) and maintained in T-Medium (Invitrogen). Wild-type and HDAC6-knockout MEF cells were generated as previously described (52) and kindly provided by Dr. Tso-Pang Yao (Duke University, Durham, NC); MEF cells were maintained in DMEM (Invitrogen). Cells were transfected via Lipofectamine 2000 (Invitrogen) in OPTI-MEM (Invitrogen), and after 4 h the medium was changed to phenol red-free RPMI with 10% double charcoal-stripped fetal bovine serum to ensure ligand-free conditions or the cells were treated with assigned condition. Nuclei were stained with 1 μ g/ml Hoechst 33342 for 2 min at room temperature. Slides were washed in PBS and mounted onto slides using DAKO Glycergel mounting medium (DAKO Corp., Carpinteria, CA). Localization of GFP-tagged AR was determined by fluorescence microscopy using a ZeissAxioplan 2 microscope (Carl Zeiss, Inc., Thornwood, NY). Transfected cells were categorized as having nuclear localization when nuclear fluorescence was greater than cytoplasmic fluorescence. Conversely, when cytoplasmic fluorescence was greater than nuclear fluorescence, cells were labeled as having cytoplasmic localization. When the fluorescent intensities of the nucleus and cytoplasm were indistinguishable, the cells were categorized as having even distribution.

Western blot analysis and immunoprecipitation

After the designated treatments, cells were lysed in modified radioimmune precipitation assay buffer [50 mM Tris (pH 7.4), 1% Igepal CA-630, 0.25% Na-deoxycholate, 150 mM NaCl, 1 mM EDTA (pH 8.0), 1 mM NaF, 2 mM phenylmethylsulfonylfluoride, 1 mM Na₃VO₄, and protease inhibitor cocktail (Sigma)]. Xenograft tumor tissues were lysed in modified radioimmune precipitation assay buffer using a homogenizer. Western analysis of HDAC6, HDAC10, AR, Hsp90 α/β , PSA, and GAPDH were as described previously (28). To immunoprecipitate Hsp90, cell lysates (200 μ g) and anti-Hsp90 monoclonal antibody (5 μ g, SPA-830; Stressgen Biotechnologies, Victoria, British Columbia, Canada) were incubated for 1 h at 4 C. Protein A/G PLUS-Agarose beads (30 μ l) were then added, and the mixture was incubated overnight at 4 C. Eluted proteins were electrophoresed and immunoblotted using anti-Hsp90, acetylated-lysine antibodies. GAPDH served as a loading control.

Real-time RT-PCR

Total RNA was prepared using the RNeasy mini kit (QIAGEN, Valencia, CA). A total of 2 μ g RNA was used to synthesize cDNA using the SuperScript III First-Strand Synthesis System (Invitrogen). Primer pairs and probes for the amplification of PSA and GAPDH are as follows: AR forward, 5'-AAGCTGCAAGGTCTTCTTCAA-3'; reverse, 5'-TCCTTCGGAATTTATCAATAGTGC-3'; and probe, 5'-6FAMAACAGAAGTAC-

CTGTGCGCCAGCATAMRA-3'. PSA forward: 5'-CATCAG-GAACAAAAGCGTGA-3'; reverse, 5'-AGCTGTGGCTGAC-CTGAAAT-3'; and probe, 5'-6FAMCAGCAGCCTGTTTCA-TCCTGAAGACACATAMRA-3'. GAPDH forward, 5'-CATGTTCGTCATGGGTGTGA-3'; reverse, 5'-GGTGCTA-AGCAGTTGGTGGT-3'; and probe, 5'-6FAMACAGCCT-CAAGATCATCAGCAATGCCTCTAMRA-3'. Real-time PCRs were performed using Premix EX Taq (Takara Bio, Inc., Shiga, Japan) with a thermal profile of 50 C for 2 min, 95 C for 10 min followed by 40 cycles of 95 C for 15 sec and 60 C for 1 min. All reactions were done in triplicate and with no reverse transcriptase or no template as negative controls. The levels of PSA were normalized to GAPDH.

HDAC6 knockdown

To generate the HDAC6-shRNA vector, we cloned the oligonucleotide 5'-GGATGGATCTGAACCTTGAGA-3' into the Lentivirus pSIH-H1-copGFP shRNA vector, which expresses GFP as a marker (System Biosciences, Mountain View, CA). This oligonucleotide targeted codons 218–237 in HDAC6 mRNA (32) and contained a sense and antisense portion separated by a hairpin loop (5'-CTTCCTGTCAGA-3'). The pSIH-H1-shLuc-copGFP vector expressing a shRNA targeting Firefly Luciferase (System Biosciences) served as a shRNA control (C4-2/shControl). Lentivirus vectors were packaged and cells infected with virus. After 3 d, cells expressing GFP (C4-2/shHDAC6) were FACS sorted, and those with the greatest reduction in HDAC6 expression were used for further studies.

Sorting of transiently transfected cells and CellsDirect one-step quantitative RT-PCR (qRT-PCR)

C4-2 cells were transfected with the control shRNA vector or cotransfected with HDAC6-shRNA and a wild-type or codon-switched HDAC6 expression vector in a molar ratio of 1:5. After 4 h, cells were cultured in ligand-free medium for an additional 16 h. Cells were then sorted based on GFP expression. PSA mRNA was measured in GFP-positive cells using the CellsDirect One-Step qRT-PCR Kit (Invitrogen). We used GAPDH to normalize PSA levels.

Immunofluorescence

HDAC6-knockdown and shRNA-control C4-2 cells were cultured on chamber slide (Nunc, Rochester, NY) for 24 h in ligand-free conditions. Then cells were cultured with or without 1 nM DHT for an additional 16 h. Cells were washed with cold PBS, fixed with 100% cold methanol for 10 min, permeabilized with 0.5% Triton X-100, and incubated in a humidifying chamber with blocking solution (5% goat serum in PBS). Next, slides were incubated with the anti-AR antibody at a 1:200 dilution in blocking solution for 1 h at room temperature. After a series of PBS washes, cells were incubated with tetramethylrhodamine isothiocyanate-conjugated antirabbit IgG diluted 1:200 for 30 min at room temperature. Nuclei were stained with 1 μ g/ml Hoechst 33342 for 2 min at room temperature. Slides were washed in PBS and mounted onto slides using DAKO Glycergel mounting medium. Localization of endogenous AR was assessed by fluorescence microscopy as described above.

Cell growth and colony formation assay

To compare proliferation of wild-type and HDAC6-knockdown C4-2 cells, exponentially growing cells were seeded at

density of 5×10^4 per well in six-well plates containing 2 ml T-medium. Cell number was counted at d 2, d 4, and d 6 after being seeded using a Coulter particle counter (Beckman Coulter, Inc., Fullerton, CA). For colony formation assays, 1×10^4 HDAC6-knockdown or shRNA-control cells were seeded onto 10-cm tissue culture dishes with medium containing various concentrations of DHT. The surviving cells were stained 2 wk later with 0.5% crystal violet, and colonies with a diameter larger than 0.5 mm were counted. Each cell growth and colony formation assay was carried out in triplicate and repeated three times.

Prostate xenograft tumor model

Athymic BALB/c male mice (Charles Rivers Inc., Wilmington, MA), 6–8 wk of age, were randomized into two groups ($n = 12$) and castrated or sham castrated. One week after surgery, each group of mice was randomized into two subgroups ($n = 6$). Then 0.25 ml of C4-2/shHDAC6 or C4-2/shControl (1×10^6) cells mixed 1:1 with matrigel (Invitrogen) was injected sc the flank of each mouse. Tumors were measured with a caliper weekly until the diameter reached 2 cm. Tumor volume was calculated using the formula $L \times W^2 \times 0.52$, where L is the larger diameter and W is the smaller diameter. Tumor take rate was calculated 12 wk after injection. All animal studies were conducted in accordance with the University of Pittsburgh Institutional Animal Care and Use Committee guidelines.

Statistics

All results are presented as mean \pm SEM or mean \pm SD. Comparisons were made with unpaired Student's t tests. GraphPad Prism software (GraphPad Software, Inc., San Diego, CA) was used in all analyses. $P < 0.05$ was considered statistically significant.

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